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Abstract

The interest in donkey milk (DM) is growing because of its functional properties and nutritional value, especially for children with allergies and food intolerances. However, most of the available reports of DM microbiota are based on culture-dependent methods to investigate food safety issues and the presence of lactic acid bacteria (LAB).

The aim of this study was to determine the composition of DM bacterial communities using a high-throughput sequencing (HTS) approach.

Bulk milk samples from Italian donkey dairy farms from two consecutive years were analysed using the MiSeq Illumina platform. All sample reads were classified into five phyla: *Proteobacteria, Firmicutes*, *Bacteroidetes*, *Actinobacteria,* and *Verrucomicrobia*. The most prevalent genera—*Pseudomonas, Ralstonia, Acinetobacter*, *Cupriavidus*, *Citrobacter* and *Sphingobacterium*—were gram-negative bacteria.

The core microbiota was composed of genera that comprise commonly associated milk bacteria, LAB and species normally found in soil, water and plants. Reads assigned to LAB genera—*Streptococcus, Lactococcus, Enterococcus, Leuconostoc, Lactobacillus,* and *Carnobacterium*—corresponded on average to 2.55% of the total reads per sample. Among these, the distribution of reads assigned to coccus- and bacillus-shaped LAB was variable between and within the farms, confirming their presence and suggesting a complex population of these bacteria in DM.

The present study represents a general snapshot of the DM microbial population, underlining its variability and motivating further studies for the exploitation of the technological potential of bacteria naturally present in DM.

Keywords:

donkey milk, bacterial communities, high-throughput sequencing

Highlights:

Bulk milk samples of donkey milk were studied with a HTS approach.

Microbial population of DM is complex, diverse, variable

The most prevalent genera are Gram negative bacteria.

1. Introduction

Donkey milk (DM) has recently received growing interest since it has been reported to be an adequate replacement for children with cow milk protein allergy, mainly due to its tolerability, nutritional contents and good taste (Monti et al., 2012). In fact, studies have demonstrated a number of qualities that make DM more favourable than cow milk: better digestibility (Tidona et al., 2011), lower allergenicity (Vincenzetti et al., 2008) and a set of unique nutritional and physicochemical characteristics (Guo et al., 2007).

Following the growing demand for DM, several new dairy farms have opened in the last few years. Italian donkey dairies are generally small, with 20 to 25 milking jennies and one or two stallions; their overall average daily production is approximately 2,000 litres, for a total of 700,000 litres per year (Milonis and Polidori, 2011). The production is mainly used for direct human consumption, while a smaller part is destined for the cosmetics and food industries. Pasteurized donkey milk is usually sold directly from the farms. However, considering its target consumers and nutritional properties, it can be sold raw, with 3 days of shelf life (similar to raw bovine milk) (Giacometti et al., 2016).

The composition of DM is closer to human milk than to cow milk and has been fully described (Salimei and Fantuz, 2012). It contains high levels of lactose and essential amino acids (Guo et al., 2007) as well as low concentrations of β-lactoglobulin and casein—the most common allergens in cow milk (Vincenzetti et al., 2008). One of the main characteristics of DM is its high concentration of lysozyme: from 1300 to 4000 mg/l, compared to 0.09 mg/l in cow milk and 40–200 mg/l in human milk (Carminati et al., 2014; Chiavari et al., 2005; Vincenzetti et al., 2008). This enzyme has bactericidal properties; it hydrolyses the murein of bacterial cell walls, causing lysis of sensitive bacteria (Chiavari et al., 2005). Currently, there is no confirmed hypothesis as to why DM is so rich in lysozyme, but it seems to positively affect the animals, defending against infections in both the mammary gland and the foal. In

addition to lysozyme, DM lactoferrin concentration is twice as high as in bovine milk (Malacarne et al., 2002), and other components have been described, such as immunoglobulins, free fatty acids and members of the lactoperoxidase peroxide system (Zhang et al., 2008), that might act synergistically against specific bacteria (Šarić et al., 2012). Traditional microbiological tests and biomolecular culture-dependent methods have been used to study the bacterial population of DM, mainly focusing on hygienic conditions and/or the presence of lactic acid bacteria (LAB) (Cavallarin et al., 2015; Pilla et al., 2010; Zhang et al., 2008; Šarić et al., 2012). Moreover, in the last few years, culture-independent methods, based on the direct analysis of DNA without a culturing step, have also been used to characterize the milk of different species (Quigley et al., 2013). PCR-denaturing gradient gel electrophoresis (PCR-DGGE), for example, has been successfully applied to the study of the microbiota of milk and dairy products (Delgado et al., 2013). However, limitations in the resolution still need to be overcome, especially for the analyses of matrices with diverse microbial communities (Ogier et al., 2004). Recently, rapid developments of high-throughput sequencing (HTS) methods have allowed a deeper and more precise evaluation of the milk microbiota from different animals, including cattle, goat, sheep, buffalo and humans (Quigley et al., 2013).

Notwithstanding the extensive literature on DM, no high-throughput analysis of its bacterial population has yet been performed, despite ever-increasing interest from both technological and commercial points of view. For this reason, the present study aimed to contribute to the knowledge of DM by characterizing its microbiota using an HTS approach.

2. Materials and Methods

2.1 Milk sampling and DNA extraction

Five donkey dairy farms (A, B, C, D, E) in the northwest part of Italy were sampled during the spring (March) of 2013 (samples A.2013, B.2013, C.2013, D.2013, E.2013) and 2014 (samples A.2014, B.2014, C.2014, D.2014, E.2014); in the second year, an additional farm was included (F; sample F.2014). These are small dairies, with a few milking jennies, family-run and with a limited production (around one litre per day, per animal); the general characteristics of the surveyed farms are summarized in Table S1. The biochemical characterization, the shelf life and the safety of the samples have been reported in a previous work (Cavallarin et al., 2015).

Bulk milk samples from healthy jennies, collected in sterile tubes, were transported to the laboratory immediately after sampling in cool conditions and stored at -20 °C until DNA extraction. Samples were treated as reported elsewhere (Dalmasso et al., 2011), and DNA was extracted from 3 ml of milk following the manufacturer protocol of the Dneasy Blood & Tissue kit (Qiagen) and quantified with a Nanodrop 2000 (Thermo Fisher Scientific). To minimize the bias associated with single extractions, triple extractions of each sample were done in parallel and mixed in a final pool.

2.2 High-throughput sequencing

Illumina libraries were prepared following the protocol described by Dalmasso et al. (2016) with the NEXTflex 16S V4 Amplicon-Seq Kit (Bioo Scientific, Austin, USA). Briefly, the bacterial V4 region of the 16S ribosomal gene was amplified from 50 ng of DNA for each sample. The universal primers 515F and 806R tailed with Illumina barcoded adapters were used with the following touchdown PCR conditions: an initial 9 cycles (15 sec. at 95°C, 15 sec. at 68°C, 30 sec. at 72°C) and then another 23 cycles (15 sec. at 95°C, 15 sec. at 58°C, 30 sec. at 72°C). The PCR products were purified using Agencourt XP Ampure Beads (Beckman

Coulter). The quality of the final products was assessed with a Bioanalyzer 2100 (Agilent Technologies).

The samples were quantified with Qubit (Invitrogen) and pooled in equal proportions for their paired-end sequencing with Illumina MiSeq for 312 cycles (150 cycles for each paired read and 12 cycles for the barcode sequence) at IGA Technology Services (Udine, Italy). To prevent focusing and phasing problems due to the sequencing of "low diversity" libraries, 30% PhiX genome was spiked in the pooled library.

2.3 Bioinformatics and data analyses

Sequence reads were trimmed with the collection command line tools of FASTX-Toolkits (http://hannonlab.cshl.edu/fastx_toolkit/) so that the quality score for each read was above 20 with more than 50 base pairs. The PRINSEQ standalone lite version (Schmieder and Edwards, 2011) was used to check and prepare the data set for the downstream analyses.

Data were then analysed with the QIIME software, version 1.9.0 (Caporaso et al., 2012). Using the uclust method (Edgar, 2010), sequences >97% identical were considered to correspond to the same operational taxonomic unit (OTU). Representative sequences were submitted to the RDPII classifier (Wang et al., 2007) to obtain the taxonomy assignment and relative abundance of each OTU using the Greengenes 16S rDNA database v13.8 (McDonald et al., 2012).

Alpha diversity was evaluated with QIIME to obtain the rarefaction curves. A rarefaction curve shows the variation in the number of OTUs identified at a given percentage of identity as a function of the number of sequence reads obtained per sample. Ideally, an optimal coverage is identified by the plateau of the curve, which indicates that increasing the number of reads does not change the number of OTUs that can be determined.

Moreover, Good's coverage (a sampling completeness indicator that indicates what percent of the total species is represented in the sample), Chao1 and ACE (richness estimators that calculate an approximate number of species in the samples using different methods), and Shannon and Simpson indices (estimators of the samples' diversity taking into account the approximated number of species and how evenly they are distributed) were determined.

Beta diversity was evaluated with the UniFrac method. Weighted UniFrac distance matrices and OTU tables were used to plot the principal coordinate analysis (PCoA) and to perform Adonis and Anosim statistical tests with the compare_category.py script of QIIME to evaluate differences between the farms, their practices and their characteristics.

The core microbiota of the samples was obtained with the compute_core_microbiome.py script in QIIME; OTUs present with more than 0.001% of the reads of each sample, in at least 9 samples, were included. The pseudo-heatmap was plotted with the gplots package in the R environment (http://www.r-project.org) using the OTUs table generated by QIIME.

3. Results and Discussion

3.1 Characteristics of the sequencing data

We obtained a total of 5,225,689 raw sequences; after filtering, 3,743,291 high-quality 16S rRNA gene sequences with an average length of 288 bp were recovered. Table 1 shows the number of analysed reads per sample. The rarefaction curves of our data (Figure S1) suggest a sufficient coverage; this consideration is further supported by the observed values of the Good's coverage estimator -higher than 0.99- for all the samples (Table 1).

3.2 Bacterial composition of donkey milk

The sequences obtained from all the studied samples correspond to five phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* (Table 2) in

agreement with the main taxons found in raw milk from different animals (Dalmasso et al. 2016; Quigley et al., 2013). The total reads corresponded to 201 families and 314 different genera (data not shown).

The most abundant genera observed in all the studied samples were gram-negative bacteria: *Pseudomonas, Ralstonia, Cupriavidus, Acinetobacter, Citrobacter* and *Sphingobacterium* (Figure 1, Table 2).

However, only the genus *Pseudomonas* reached high percentages in almost all the studied samples. Furthermore, previous studies, using culture-dependent methods, had found that *Pseudomonas* spp. is an important component of the DM microbiota (Cavallarin et al., 2015; Giacometti et al., 2016). This observation is consistent with a previous report that indicated *Pseudomonas* spp. to be the predominant microorganism in different milks (Quigley et al., 2013); in raw bovine milk stored at low temperatures, *Pseudomonas* spp. may constitute up to 70-90% of the total microbial population (Sørhaug and Stepaniak, 1997). The abundance of these microorganisms, which are the most common cause of milk spoilage (Ercolini et al., 2009), mainly because of their proteolytic activity and psychrotolerant nature, leads to the short commercial shelf life of the product (3 days). Given that raw DM is sold, is necessary to focus attention not only on spoilage but also on hygienic safety. Cavallarin et al., (2015), while characterizing DM by traditional microbiological methods, showed the absence of pathogens. In our study, the limitations of the analytical approach (genus identification and the impossibility of viability evaluation) did not allow us to infer the hygienic safety status.

The other genera (*Ralstonia, Cupriavidus*, *Acinetobacter*, *Citrobacter* and *Sphingobacterium*) (Figure 1, Table 2), are considered environmental microorganisms since they are commonly found in soil, water and dust. *Ralstonia* spp. and *Cupriavidus* spp. are phylogenetically related to *Pseudomonas* spp., and they have only recently been reclassified (Balkwill, 2015;

Yabuuchi et al., 2015). Nevertheless, HTS studies have found them in human, bovine, goat and buffalo milk (Quigley et al., 2013).

The composition of the DM core microbiota, i.e., those OTUs shared between the samples, was also evaluated. This core contained 4 families and 24 genera that comprise commonly associated milk bacteria, LAB and species normally found in soil, water and plants (Figure 2). One compelling member of the core was the genus *Akkermansia* since the only species that currently forms the genus, *Akkermansia muciniphila*, has been linked with intestinal health, the metabolic status of obese and diabetic patients, and markers of inflammation and immune responses (Reunanen et al., 2015). This potential probiotic bacterium uses mucin—a protein amply present in milk—as its main source of carbon and nitrogen and has been detected in human and animal gut environments (Belzer and de Vos, 2012), including in donkeys (Liu et al., 2014). Additionally, this bacterium has been detected in breast milk using real-time PCR (Collado et al., 2012), and just recently, Ottman (2015) reported its ability to grow in human milk. Further studies are needed to isolate and characterize the probable *Akkermansia* species present in DM; nonetheless, our observation creates a new perspective on this functional microbe that has not yet been isolated from food matrices.

Subsequently, we analysed the differences in the distribution of the OTUs between and within the farms, where some particular trends were observed. Beta diversity analyses, using the UniFrac method, were performed to compare the samples between the dairies. We performed Anosim and Adonis tests for all the different parameters of the dairies (farm area, altitude of the farm, breed, milking practice, farming type and feeding), but none of them had a significant ($P>0.01$) influence on the variation observed in the DM microbiota (data not shown). The only variable that resulted in significant differences was the sampling year, indicating that the bacteria present in the samples from 2013 were different from those from 2014 (Figure S2 of the supplementary material). This very interesting result suggests that the

variability in the milk microbiota may derive from the individual components of each animal and/or their lactation period. As the gestation period in donkeys is approximately one year and jennies produce milk only for 6 months, we sampled milk from completely different animals in each year. Moreover, the different stages of lactation of the milking jennies in each farm would further contribute to the variability observed. These interindividual differences have been amply described for breast milk (Cabrera-Rubio et al., 2012), and we can most likely assume that they are also valid for other mammal milks; still, further studies are needed to corroborate this presumption.

Additionally, the Chao1 richness estimator and the Shannon diversity index of Farm D (Table 1) and its rarefaction curves (Figure S1) demonstrated that this farm had the fewest number of observed genera of all the tested farms. In particular, the most representative were *Ralstonia* and *Cupriavidus* spp. (Figure 1). This low variability could be a consequence of the farming practices since it is the only sampled farm run extensively; the animals are free to pasture and are hand milked only when it is requested (Table S1). Moreover, Cavallarin et al. (2015) showed that the samples from this dairy had lower total bacterial counts than those milked automatically. This thesis could be further confirmed by i) the higher percentage of *Streptococcus* spp. reads (Figure 3A), a genus considered skin-associated (Cogen et al., 2007), and ii) the low percentage of *Pseudomonas* spp. reads (Figure 3B); members of this genus are normally present in water, and they might derive from the water used to rinse the milking machinery. The supposition that farm practices have a direct consequence in the milk microbiota has also been supported by goat farm observations, where hand milking practices resulted in lower total bacteria counts (Delgado-Pertiñez et al., 2003).

3.3 Lactic acid bacteria in donkey milk

Studies regarding the microbiota of DM have focused on the hygienic quality of DM (Pilla et al., 2010; Zhang et al., 2008; Šarić et al., 2012). Only more recently have some authors characterized the lactic bacteria for their probiotic activity and potential technological aspects (Carminati et al., 2014; Soto del Rio et al., 2016). It is generally accepted that LAB are the dominant population in milk from several species, independent of the methodology used for study. Reports with an HTS approach in cow, sheep, buffalo and human milk have identified LAB reads that corresponded to more than 40% of the total sequences (Quigley et al., 2013).

In our samples, we detected reads for the LAB genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus* (Figure 3A, Table 2) with an average of 2.55%, ranging from 0.02% (zoomed in Figure 3B) to 15.85%, of the total reads per sample, which is consistent with the low LAB count in these samples reported by Cavallarin et al., (2015).

In this study, all the samples had sequences that corresponded to both coccus (*Enterococcus, Lactococcus,* and *Streptococcus*)- and bacillus (*Carnobacterium, Lactobacillus,* and *Leuconostoc*)-shaped genera (Figure 3), although in different proportions. This result is in contrast with other studies, where the authors isolated and characterized only coccus-shaped LAB (Carminati et al., 2014) or bacilli species (Soto del Rio et al., 2016). However, there was important variability in the distribution of cocci/bacilli reads both within and between the different farms (Figure 3). In particular, cocci were noticeably present only in Farms C and E in both sampling years (Figure 3C), whereas sample D.2013 presented more cocci reads. Sample A.2014 was characterized by a similar proportion of bacilli and cocci reads. Regarding the bacilli, the sole sampling year of Farm F showed only bacilli reads (Figure 3C), while in Farm B, their presence was not constant; in 2013, the prevalence of cocci was clear, while the situation was reversed in the following year. It is relevant to note that these two

bacilli-rich samples (B.2014 and F.2014) were the ones that had higher percentages of LAB reads from the total number of sequences (Figure 3A).

These results are relevant to the possible production of probiotic milks. Several authors have proposed novel fermented DM beverages that used lactobacilli strains isolated from bovine milk adapted to grow in DM (Chiavari et al., 2005; Perna et al., 2015). Consequently, having available bacilli strains naturally adapted to DM might be notable from a biotechnological point of view to facilitate the production of these beverages.

Overall, the results suggest that the LAB population of DM is complex, diverse, variable and may depend upon several parameters, thus requiring further investigation.

4. Conclusions

The present survey provides a broad characterization of the bacterial composition of DM, allowing a description of microorganisms not previously detected in this product. The microbiota of DM is mainly composed of gram-negative bacteria. Unlike other milks, LAB reads were present in low percentages, both cocci and bacilli, even though their growth is not particularly favoured by the composition of DM. The HTS analysis of diverse farms allowed the proposal of several genera as members of a core DM microbiota. The observed results also support the premise that the microbial composition of DM may be influenced by individual animal components.

The present study aimed to give a general picture of the bacterial communities present in DM, and it has shown that this microbiota can be highly diverse. Further studies are needed to better understand the dynamics between the bacterial population in this matrix and the relationship between the milk components.

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Figure 1. Distribution of the most abundant genera in donkey milk. Percentages refer to the total number of reads per sample.

Figure 2. Core microbiota of donkey milk. **A)** Taxonomic distribution of the OTUs present at > 0.001% in at least nine samples. **B)** Pseudo-heatmap of the distribution (%) of the core OTUs. Samples were clustered using Euclidean distance and the complete method.

Figure 3. Distribution of lactic acid bacteria detected in donkey milk samples. **A)** Abundance of LAB genera found in the studied samples; percentages refer to the total number of reads. **B)** Zoomed-in for the lower percent levels of LAB genera abundance in each sample **C)** Relative abundance for the sum of the percentages of coccus-shaped (*Enterococcus, Lactococcus, Streptococcus*) and bacillus-shaped (*Carnobacterium, Lactobacillus, Leuconostoc*) LAB genera reads for each farm.

Table 1. Numbers of sequences analyzed, observed OTUs, coverage and diversity estimators for all the studied samples.

Table 2. Percentages of the most abundant taxonomical groups of the sampled donkey milk farms.

Figure S1. Rarefaction curves of the observed species for each studied sample.

Figure S2. Principal coordinate analysis (PCoA) of the surveyed donkey milk samples. The plot was based on the weighted UniFrac distance matrix of the microbiota. The dots and names in red correspond to the sampling of 2013, while the blue ones correspond to 2014.

Table S1. General characteristics of the surveyed donkey milk farms. Modified from (Cavallarin et al., 2015)

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Table 1

Sample	Reads	Good's coverage	Observed OTUs	Chao 1	ACE	Shannon	Simpson
A.2013	294,557	0.994	5078	6875.19	6833.26	7.09	0.96
A.2014	188,349	0.993	3760	5616.95	5513.89	6.15	0.92
B.2013	203,091	0.993	4008	5410.35	5345.92	6.52	0.94
B.2014	223,728	0.993	4338	6002.83	6138.72	5.46	0.81
C.2013	279,374	0.993	5745	7880.04	7818.74	7.37	0.97
C.2014	850,529	0.998	7686	9465.44	9477.87	6.60	0.92
D.2013	172,717	0.996	2316	2965.35	2964.90	5.65	0.90
D.2014	220,559	0.997	2019	2928.77	2853.42	2.90	0.46
E.2013	254,323	0.994	3839	5474.76	5453.59	5.73	0.87
E.2014	501,861	0.997	5012	6989.73	7026.50	5.99	0.92
F.2014	554,203	0.997	5759	7702.27	7826.34	5.84	0.89

Table 2

Phylum	Genus	Farms										
		A.2013	A.2014	B.2013	B.2014	C.2013	C.2014	D.2013	D.2014	E.2013	E.2014	F.2014
Actinobacteria		**0.18**	**0.33**	**2.00**	**0.38**	**2.04**	**0.22**	**2.17**	**0.17**	**0.31**	**0.02**	**0.40**
	Arthrobacter	0.01	0.001	0.19	0.02	0.07	0.001	0.004	0.02	<0.000	0.001	0.17
	Kocuria	<0.000	0.01	0.01	0.004	0.69	0.04	0.001	0.002	ND	ND	0.001
	Corynebacterium	0.004	0.01	0.53	0.18	0.08	0.08	0.04	0.02	0.02	0.005	0.05
	Pseudonocardia	0.01	0.002	0.05	0.004	0.12	ND	0.39	0.004	0.02	<0.000	0.001
	Rothia	0.001	0.001	0.21	0.003	0.01	0.004	0.01	0.001	0.003	0.001	0.001
Bacteroidetes		**24.15**	**2.52**	**1.00**	**0.93**	**0.95**	**1.37**	**0.81**	**0.05**	**0.60**	**0.70**	**0.78**
	Chryseobacterium	3.42	1.31	0.002	0.002	0.36	0.36	0.01	0.005	0.01	0.002	0.23
	Cloacibacterium	0.02	0.002	0.21	0.004	0.19	<0.000	0.64	<0.000	0.09	<0.000	<0.000
	Flavobacterium	3.00	0.31	ND	0.001	0.01	0.05	0.01	0.001	0.48	0.58	0.23
	Sphingobacterium	17.34	0.70	0.16	0.69	0.33	0.88	0.004	0.01	<0.000	0.02	0.11
Firmicutes		**0.43**	**0.93**	**8.09**	**17.39**	**2.59**	**0.76**	**6.38**	**0.33**	**0.89**	**0.08**	**9.80**
	Carnobacterium	ND	0.002	0.002	0.003	0.002	0.004	0.001	0.01	<0.000	0.002	7.32
	Enterococcus	0.005	0.02	0.01	0.001	0.32	0.32	0.01	<0.000	0.001	ND	0.002
	Lactobacillus	0.03	0.21	0.04	3.16	0.05	0.01	0.02	0.01	0.003	0.002	0.003
	Lactococcus	0.07	0.03	0.65	1.01	0.06	0.08	0.04	ND	0.01	ND	0.001
	Leuconostoc	0.001	0.001	0.06	11.61	0.01	0.004	0.002	ND	0.001	<0.000	0.02
	Streptococcus	0.05	0.07	0.40	0.08	0.16	0.02	1.98	0.02	0.03	0.02	0.05
	Veillonella	0.04	0.31	0.16	0.03	0.01	0.01	1.98	0.01	0.003	0.01	0.02
Proteobacteria		**74.92**	**91.09**	**87.86**	**75.54**	**93.99**	**94.01**	**89.64**	**93.89**	**98.05**	**92.13**	**84.85**
	Acinetobacter	2.39	1.72	3.52	2.21	4.19	23.36	4.03	0.03	0.80	0.02	0.37
	Agrobacterium	0.06	0.28	0.02	0.003	0.04	0.11	0.001	0.003	<0.000	0.10	0.01
	Citrobacter	0.27	0.07	0.002	0.03	5.95	3.75	0.01	0.01	0.001	0.02	14.00
	Cupriavidus	ND	0.002	ND	6.57	ND	0.79	ND	86.96	ND	0.002	0.002
	Janthinobacterium	2.57	0.001	0.001	0.002	<0.000	0.07	0.003	0.03	<0.000	3.83	3.78
	Mesorhizobium	0.004	ND	0.04	ND	0.04	ND	0.08	0.001	0.02	ND	<0.000
	Mycoplana	0.14	0.17	0.001	<0.000	0.001	0.7	0.01	<0.000	0.001	0.01	0.001
	Ochrobactrum	0.03	0.02	0.001	0.001	0.31	0.37	ND	ND	ND	ND	<0.000
	Pseudomonas	54.48	84.22	24.18	57.96	25.52	24.70	0.11	0.26	72.57	76.19	59.53
	Ralstonia	4.18	ND	42.30	ND	28.65	ND	60.68	0.002	16.34	<0.000	0.001
	Stenotrophomonas	5.20	0.41	1.24	3.54	2.25	1.23	0.002	0.004	ND	0.02	0.004
	Sphingomonas	0.03	0.07	0.49	0.20	0.23	0.18	0.57	0.31	0.06	0.07	0.04
	Yersinia	0.01	0.001	ND	0.55	2.67	1.12	ND	0.002	ND	0.25	0.02
Verrucomicrobia		**0.14**	**0.11**	**0.17**	**0.07**	**0.02**	**0.11**	**0.07**	**0.01**	**0.01**	**0.001**	**0.03**
	Akkermansia	0.001	0.01	0.09	0.02	0.01	0.002	0.01	0.01	0.001	<0.000	0.02

ND stands for non detected reads in the sample for that particular taxon

Table S1

	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F
Farm area (ha)	35	12	10	10	42	20
Altitude above sea level (m)	194	1110	395	600	183	430
Jennies [a] (no.)	45	40	40	70	32	32
Milking jennies [a] (no.)	7-10	7-10	8-10	30-33	6-10	6-10
Herd breed	Crossbreds	Martina Franca	Crossbreds	Crossbreds	Martina Franca, Ragusana, Crossbreds	Crossbreds
Milking practice	Automatic in milking room	Automatic in milking room	Automatic in cowshed	Hand milking	Automatic in milking room	Automatic in in milking room
Farming type	Semi-extensive	Semi-extensive	Semi-extensive	Extensive	Semi-extensive	Semi-extensive
Feed	Grazing - Hay	Hay - Bread – Protein supplementation	Grazing - Hay	Grazing - Hay	Grazing - Hay	Grazing - Hay
Milk use	Food - cosmetics	Food	Food - cosmetics	Food - cosmetics	Food - cosmetics	Cosmetics

[a] counted during the visits

Figure 1

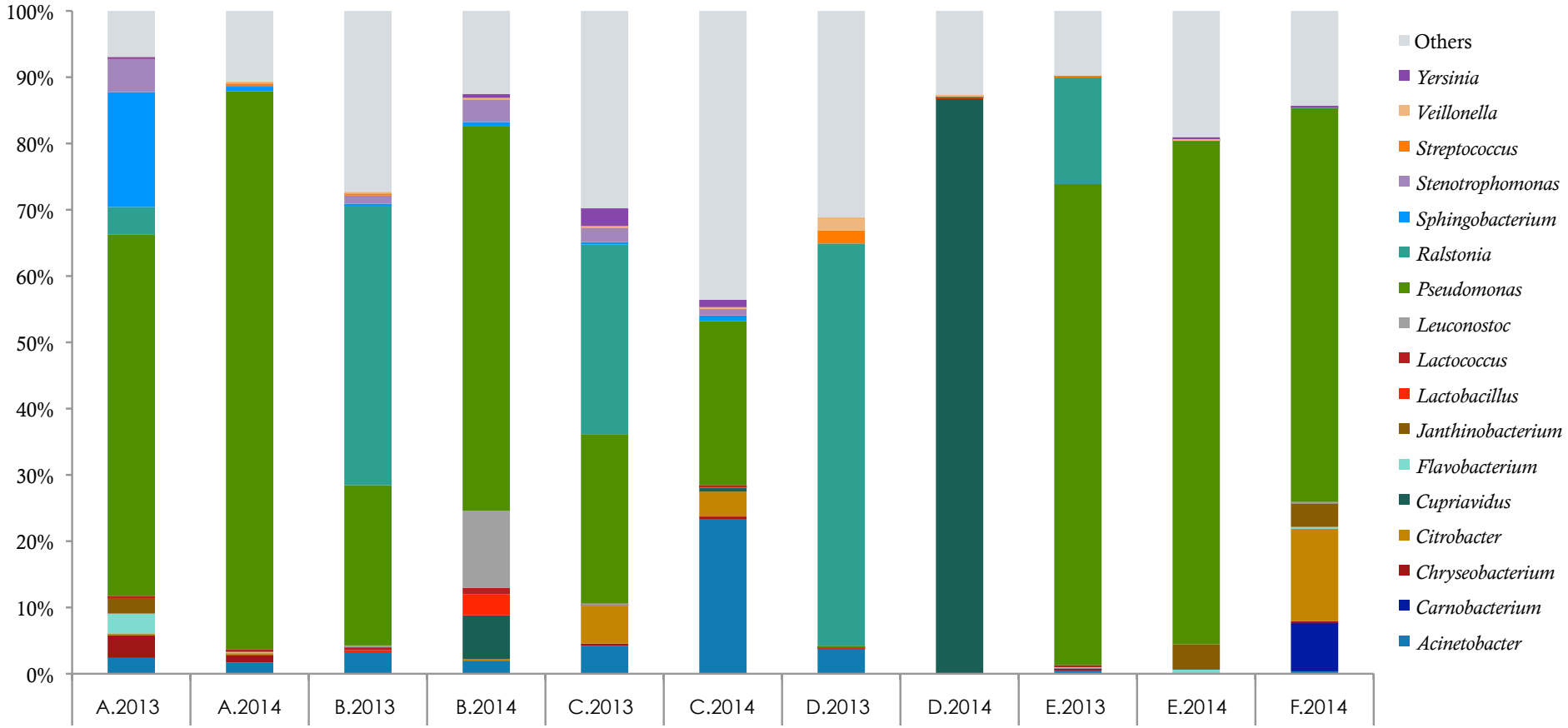

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A.2013
A.2014
B.2013
B.2014
C.2013
C.2014
D.2013
D.2014
E.2013
E.2014
F.2014
Others
Yersinia
Veillonella
Streptococcus
Stenotrophomonas
Sphingobacterium
Ralstonia
Pseudomonas
Leuconostoc
Lactococcus
Lactobacillus
Janthinobacterium
Flavobacterium
Cupriavidus
Citrobacter
Chryseobacterium
Carnobacterium
Acinetobacter

Figure 2

A)

Phylum	Class	Order	Family	Genus
			Actinomycetaceae	*Actinomyces*
			Corynecateriaceae	*Corynebacterium*
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	*Arthrobacter*
				Rothia
			Mycobacteriaceae	*Mycobacterium*
			Nocardiopsaceae	
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Weeksellaceae*	*Cloacibacterium*
Cyanobacteria	*Shingobacteriia*	*Sphingobacteriales*	*Shpingobacteriaceae*	*Sphingobacterium*
	Chloroplast	*Streptophyta*		
		Bacillales	*Bacillaceae*	*Bacillus*
			Staphylococcaceae	*Staphylococcus*
Firmicutes	*Bacilli*	*Gemellales*	*Gemellaceae*	
		Lactobacillales	*Lactobacillaceae*	*Lactobacillus*
			Streptococcaceae	*Streptococcus*
	Clostridia	*Clostridiales*	*Veillonellaceae*	*Veillonella*
			Bradyrhizobiaceae	*Bradyrhizobium*
	Alphaproteobacteria	*Rhizobiales*	*Methylobacteriaceae*	*Methylobacterium*
			Phyllobacteriaceae	
			Rhizobiaceae	*Agrobacterium*
		Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas*
			Comamonadaceae	*Acidovorax*
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Janthinobacterium*
		Neisseriales	*Neisseriaceae*	*Neisseria*
				Citrobacter
		Enterobacteriales	*Enterobacteriaceae*	*Escherichia*
	Gammaproteobacteria	*Pasteurellales*	*Pasteurellaceae*	
			Moraxellaceae	*Acinetobacter*
		Pseudomonadales	*Pseudomonadaceae*	*Pesudomonas*
Verrucomicrobia	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Akkermansia*

B)

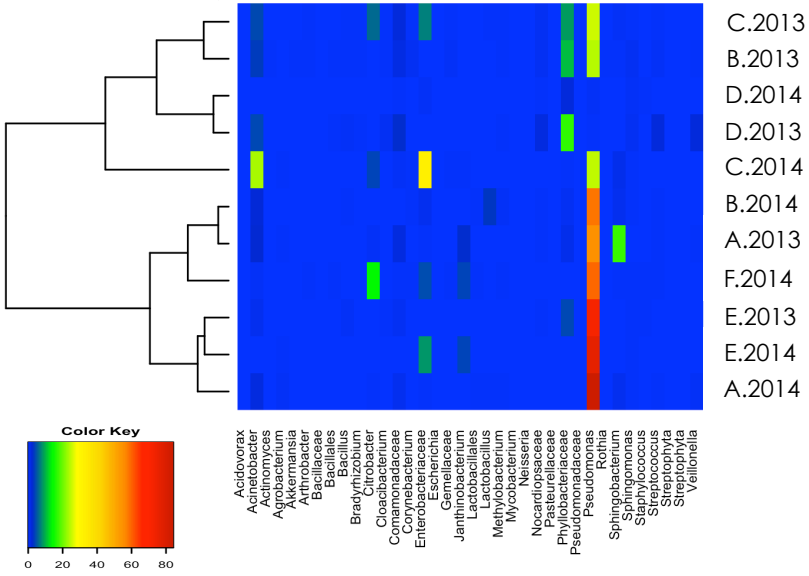


Figure 3

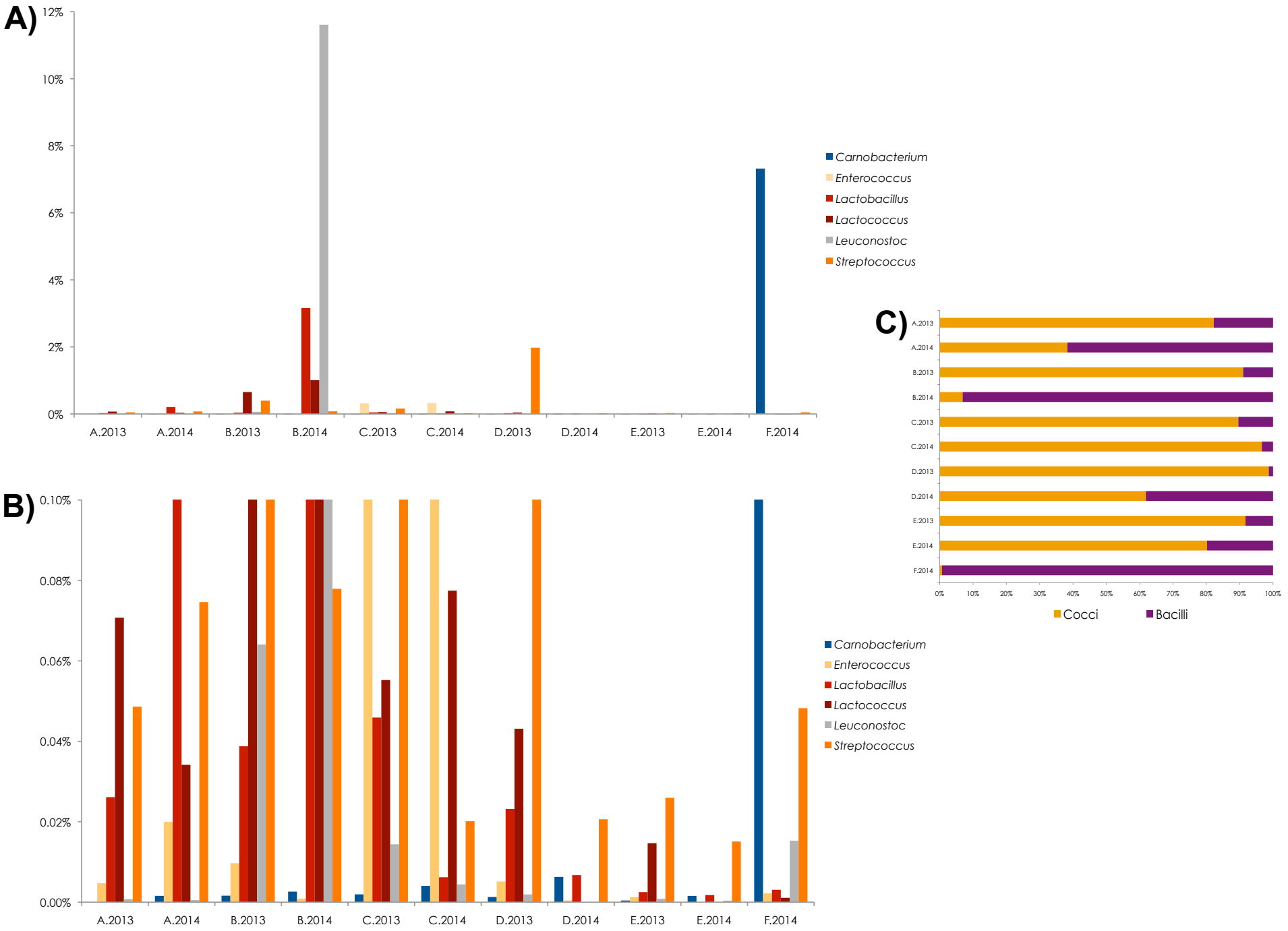

A)
12%
10%
8%
6%
4%
2%
0%
A.2013
A.2014
B.2013
B.2014
C.2013
C.2014
D.2013
D.2014
E.2013
E.2014
F.2014
Carnobacterium
Enterococcus
Lactobacillus
Lactococcus
Leuconostoc
Streptococcus
B)
0.10%
0.08%
0.06%
0.04%
0.02%
0.00%
C)
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Cocci
Bacilli

Figure S1

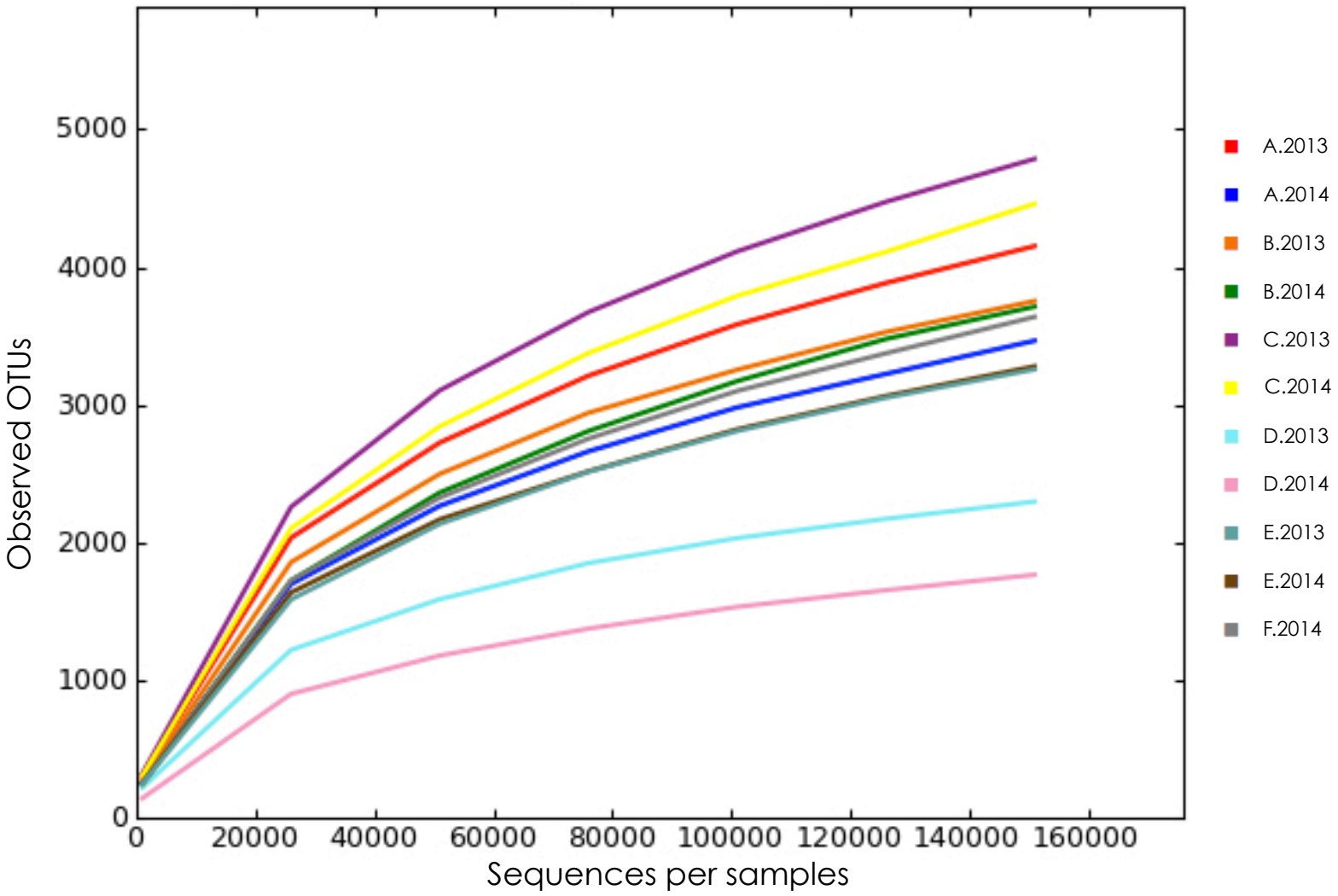
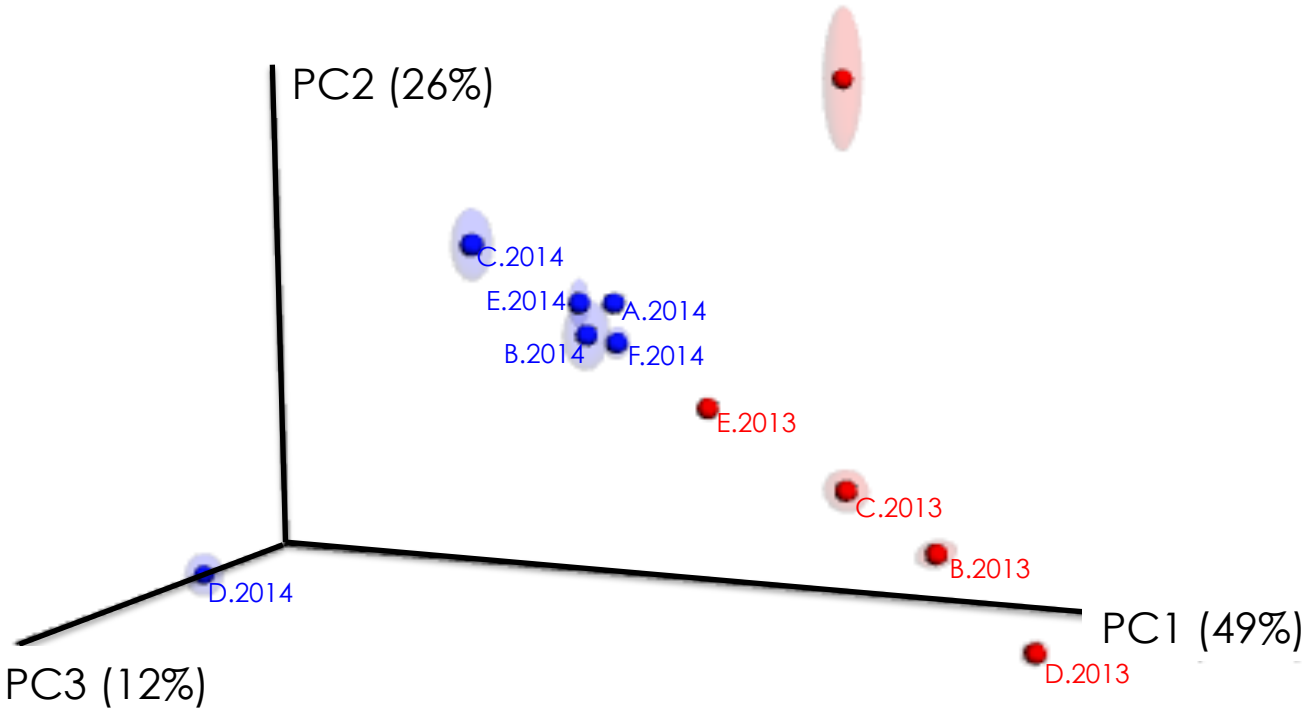

Observed OTUs
5000
4000
3000
2000
1000
0
0
20000
40000
60000
80000
100000
120000
140000
160000
Sequences per samples
A.2013
A.2014
B.2013
B.2014
C.2013
C.2014
D.2013
D.2014
E.2013
E.2014
F.2014

Figure S2


PC2 (26%)
C.2014
E.2014
A.2014
B.2014
F.2014
E.2013
C.2013
B.2013
D.2014
PC1 (49%)
D.2013
PC3 (12%)